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# Establishment success and crop growth effects of a beneficial soil fungus inoculated into Swiss corn fields

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**Keywords**

Arbuscular mycorrhizal fungi, maize, SMRT sequencing, Rhizoglomus irregulare, Phosphorous, Soil ecological engineering

**Abstract**

One major strategy to increase the sustainability of agricultural systems consists of enhancing internal ecosystem processes that support crop production and reduce external resource inputs. However, specific approaches to achieve this goal still need to be identified. Here, we investigated whether inoculation of a well-characterized strain of a plant symbiotic arbuscular mycorrhizal (AM) fungus into Swiss corn fields leads to successful establishment of the fungus in plant roots and can generate agronomic benefits for maize production.

We used single-molecule real-time (SMRT) DNA sequencing to assess community composition of native AM fungi and identified environmental factors affecting the abundance of these communities. We identified environmental, management and biological factors affecting establishment success of the introduced fungus and effects on corn yield.

Abundance and sequence richness of native AM fungal communities varied with site conditions, such as soil texture, pH, and nutrient status. While there was a negative trend of native AM fungal abundance with increasing soil P contents, we found significantly positive relationships between soil P contents and establishment success of the inoculated fungus, as well as significantly negative relationships between establishment success and abundance of native AM fungal communities. Although molecular quantification using strain-specific qPCR indicated that the inoculated strain strongly increased in abundance in roots from most soils investigated, total AM fungal root colonization was only significantly increased in one soil, indicating successful competition of the inoculant for root niche space against native AM fungi. Positive effects on corn yield were only observed when inoculation increased root colonization and were negatively correlated to P fertilization levels.

The results imply that phosphorus plays a major role in defining abundance and composition of native AM fungal communities and that these effects determine establishment success of the inoculant. The results further indicate that positive effects on crop yield may only be expected when potentially achievable root colonization levels are not yet reached.

## 1. Introduction

### *1.1 Soil life and agricultural sustainability*

The steady growth of the global human population requires increased food production. While current agricultural systems achieve high yields in many parts of the world, this is related to high environmental costs. Modern agriculture is a major cause of soil degradation, ground- and surface water eutrophication, biodiversity losses, and greenhouse gas emissions (Foley *et al.*, 2005; Amundson *et al.*, 2015; Steffen *et al.*, 2015; Tsiafouli *et al.*, 2015).

One strategy to enhance the sustainability of agricultural systems is to promote internal regulatory ecosystem processes provided by biological communities while reducing external resource inputs. This approach is termed 'ecological intensification' (Bommarco *et al.*, 2013). This approach can also be applied to soils because soil macro- and microorganisms are responsible for most soil nutrient cycling processes. They decompose organic materials, mineralize and transform them into plant available nutrient forms, therefore determining whether nutrients are taken up by plants or are more likely to be lost from the plant-soil system. 'Soil ecological engineering' has been proposed as a concept to manage the abundance and activities of soil biota to harness the ecosystem services they are able to provide with the aim to enhance the nutrient use efficiency and sustainability of agricultural systems (Bender *et al.*, 2016).

### *1.2 AM fungi in agricultural systems*

One important group of soil biota with potential to be used for soil ecological engineering are arbuscular mycorrhizal (AM) fungi. AM fungi are plant symbionts, which form intimate associations with most land plants, including many important crops. They are known for their ability to promote plant nutrition and to contribute to ecosystem sustainability. For example, up to 80% of a plants P can be provided by AM fungi (Smith and Read, 2008). They have been shown to enhance plant resistance to environmental stress, such as drought or pathogen attacks (Bowles *et al.*, 2016a; Sánchez-Bel *et al.*, 2018) , to be major players in N cycling (Hodge and Fitter, 2010) and to reduce nutrient losses through leaching and reduce N2O emissions from soil (Bender et al., 2014;

Cavagnaro et al., 2015; Storer et al., 2017). Due to their ubiquity, their contribution to crop yield and ecosystem functioning can be hard to quantify in field settings (Ryan and Graham, 2018).

Intensive agricultural management can exert adverse effects on AM fungal communities (Helgason *et al.*, 1998; Oehl *et al.*, 2003; Verbruggen *et al.*, 2010; Bowles *et al.*, 2016b). For instance, Verbruggen *et al.* (2010) found that AM fungal communities from conventionally managed arable fields were lower in diversity as compared to organically managed fields and grasslands. A study by (Oehl *et al.*, 2003) showed that increasing land-use intensity, besides reducing AM fungal diversity, also lead to shifts in AM fungal species compositions and their functional characteristics. Köhl *et al.* (2014) showed that AM fungal communities from no-till fields enhanced plant P uptake more than communities from conventionally tilled fields and Verbruggen *et al.* (2012) showed that communities in organically managed fields reduced P leaching losses from soil stronger than communities from conventionally managed fields. Therefore, negative effects of intensive management on AM fungal communities could hamper proper functioning of agro-ecosystems and could, hypothetically, contribute to the inefficient resource use found in many intensively managed arable systems (Liu *et al.*, 2010).

*1.3 AM fungal inoculation*

An alternative strategy to increase nutrient use efficiency in arable farming and to reduce environmental impacts could be to restore efficient ecosystem functioning through inoculation of AM fungi into arable fields. By selection of AM fungal isolates that are fast colonizing and efficient in scavenging for soil nutrients and enhancing plant nutrition, targeted effects on crop production and ecosystem functioning could be achieved.

However, the environmental conditions at a specific field site must be appropriate to enable an inoculated fungus to survive and to provide benefits. Different AM fungal species have been shown to thrive under different soil conditions as determined by soil texture, pH or nutrient contents, or also depending on climatic and geomorphological conditions, as well as different management practices (Oehl *et al.*, 2010; Oehl *et al.*, 2017). Similarly, these abiotic conditions

have also been shown to affect effects of inoculations on plant growth (Lekberg and Koide, 2005; Hoeksema *et al.*, 2010; Pellegrino *et al.*, 2015).

Moreover, fungi that are introduced into field sites will be competing with already established, native, fungal biota. It has been shown that higher microbial diversity diminishes the invasion success of a microbial pathogen in soil (van Elsas *et al.*, 2012). These effects have been ascribed to higher resource use of more diverse communities and therefore higher competitive pressure on the invader (Mallon *et al.*, 2014). Similarly, higher AM fungal diversity could reduce successful establishment of an inoculated AM fungus by enhancing competitive pressure for resources, such as symbiotically derived C from host plants, soil nutrients or available space for symbiotic interactions in the plant root. Therefore, AM fungi introduced into a new habitat, should be able to establish better at sites where the native AM fungal community is less competitive or less well developed (i.e. comprising low diversity and/or low abundance).

However, a less well-developed AM fungal community might indicate that the environmental conditions at a specific site are not appropriate for AM fungi in general. Therefore, an inoculated fungus might also be hindered in establishment. The relative importance of site-specific environmental factors and of the state of native AM fungal communities in determining the establishment success of an inoculated AM fungal isolate remains unclear. A recent study by Niwa *et al.* (2018) suggested that dominance of an inoculated fungus over native AM fungal communities is a prerequisite to generate yield responses in soybean. However, it remains unclear under which conditions such dominance occurs. Further insights into AM fungal community composition and the environmental and agricultural management factors affecting inoculation success with particular assessments of the establishment of inoculated AM fungal strains are necessary.

### *1.4 Study overview*

We set up a field study to test whether inoculation with a well characterized and efficiently colonizing AM fungus leads to establishment of the fungus in plant roots and affects agronomic

plant parameters. We selected 8 farmer-managed field sites spread across Switzerland on which maize was grown. We assessed soil parameters and the native AM fungal communities present in the fields. We installed experimental plots and inoculated them at the time of maize sowing, either with inoculum of the AM fungus *Rhizoglomus irregulare* or non-mycorrhizal control inoculum. At the time of harvest, we collected roots to assess AM fungal root colonization and to extract DNA for molecular analysis to quantify the establishment of the introduced fungus. Moreover, we assessed plant yield and nutrient contents. We hypothesize that (i) native AM fungal communities from different fields differ in their community composition and abundance. These differences are related to site specific characteristics (soil parameters, nutrient contents, and management practices). Furthermore, we hypothesize that (ii) the inoculated fungus establishes more successfully at sites, where native AM fungal communities are less diverse or less abundant and that (iii) at sites where the inoculated fungus establishes best, biggest effects on maize growth and/or nutrition will be observed.

## 2. Material and Methods

### *2.1 Sites*

Eight Swiss farms where selected for the trial based on a farmer's willingness to cooperate and foreseen maize cultivation for the 2015 growing season. Farmer contacts had been established during monitoring efforts of the Swiss cantonal authorities and from a farmer network established at the Plant-Soil-Interactions group at Agroscope. We selected fields from geographically distinct regions in Switzerland (Figure 1).

Directly before inoculation, soil samples were collected from each of the 12 installed plots at each site (see below) with a soil auger (diameter: 5 cm, depth 20 cm). Samples were thoroughly mixed to form a composite sample representative for each field. Soil texture, organic C, potential cation exchange capacity, soil pH, total N and P and available soil P extracted with CO2-saturated water were determined according to the reference methods of the Swiss Federal Research Stations

(Eidgenoessische Forschungsanstalten FAL, RAC, FAW, 1996). A sub-sample was frozen for later DNA extraction. Soil characteristics are given in Table 1.

***Table 1:*** *Selected soil properties of the 8 field sites investigated*

| Site | Sand (mass %) | Silt (mass %) | Clay (mass %) | organic C (mass %) | potential CEC (cmol+/kg) | pH | Total Nitrogen (mass %) | Available Soil P (mg/ kg soil) | Total Soil P (mg/ kg soil) |
|---|---|---|---|---|---|---|---|---|---|
| **Cazis (C)** | 42.1 | 48.1 | 7.9 | 1.1 | 4.4 | 8.0 | 0.108 | 0.26 | 549.7 |
| **Ecublens (E)** | 47.7 | 31.1 | 18.6 | 1.53 | 16.3 | 5.9 | 0.206 | 1.20 | 690.9 |
| **Schafisheim (SH)** | 60.8 | 21.1 | 15.6 | 1.47 | 11.8 | 6.9 | 0.165 | 2.72 | 787.9 |
| **Sins (S)** | 44.5 | 36.7 | 16.3 | 1.46 | 14.0 | 6.7 | 0.198 | 1.65 | 782.2 |
| **Unterendingen (U)** | 27.2 | 52.1 | 18.5 | 1.26 | 13.0 | 7.4 | 0.139 | 1.26 | 735.2 |
| **Wängi (W)** | 45.4 | 33.2 | 19 | 1.41 | 14.3 | 6.7 | 0.174 | 1.60 | 645.8 |
| **Vordemwald (V)** | 46 | 28.7 | 21.4 | 2.27 | 20.4 | 6.9 | 0.304 | 1.04 | 913.5 |
| **Zofingen (Z)** | 37.3 | 37.7 | 21.4 | 2.07 | 18.7 | 6.3 | 0.25 | 4.24 | 1009.3 |

*2.2 Inoculum production*

Inoculum of the fungus *Rhizoglomus irregulare* (Blaszk., Wubet, Renker & Buscot, previously named *Glomus intraradices*, isolate SAF 22, see Schlaeppi *et al.* (2016)), had been produced in the greenhouse in 7 L pots filled with an autoclaved 3:17 (v/v) soil:sand mixture supplemented with 5% inoculum and planted with *Plantago lanceolata* L.. Pots were watered regularly and received every second week 20 ml of a modified Hoagland solution (Hoagland and Arnon, 1950), containing one quarter of the original P concentration. After 3 months, watering was ceased and pots were dried out. Plants were cut on the surface and the pot content removed. Roots were cut into pieces of < 5 cm. The mixture of sand, soil, roots and AM fungal spores was used to inoculate the maize field. A control inoculum was produced similarly to the AM fungal inoculum but without adding the fungus.

*2.3 Seeding and Inoculation*

AM fungi were inoculated as soon as possible after the farmers had seeded. Twelve plots, each comprising an area of 1 $m^2$ (corresponding to 2 rows of maize plants for the length of 1 meter)

were marked in a squared 3x4 design with 1 m distance between plots. Plots were alternately assigned to control or AM fungal treatments. In each plot, maize seeds were carefully removed from soil and the complete seed furrow was dug out to a depth of approx. 20 cm and 20 cm width, comprising a soil volume of approx. 40 L. The soil was stepwise filled back into the hole and the respective inoculum (control or AM fungus) was progressively mixed in. Per furrow, 1 L of inoculum was used, resulting in 2 L of inoculum per plot. The amount of inoculum in the seed furrow corresponded to an inoculum concentration of 2.5% (v/v). Seeds were put back into the soil-inoculum mix in their former position and covered with soil. At some sites, seeds had already germinated at the time of inoculation, potentially exposing the seedling to stress.

Until harvest, the fields were managed by the collaborating farmers who provided information about the management actions applied. Management practices typical for Swiss agriculture were applied. An overview of the management actions taken is provided in Table 2. All fields were ploughed to a depth of at least 20 cm before sowing and all fields except field Z had a grass-clover mixture as preceding crop. Some farmers provided detailed nutrient concentrations of the organic fertilizers (e.g. manure and slurry) applied. Where this information was not available, standard concentrations for Swiss organic fertilizers were taken following the Swiss Principles of Fertilizer Application in Arable and Forage Cultivation (Flisch *et al.*, 2009) and used to calculate the total amounts of nutrients reported in Table 2. All fertilizer compounds containing P (organic fertilizers) were applied before or at the date of sowing and soil sampling. For 5 of the 8 sites, a fraction of the total amount of N fertilizer reported by the farmers was applied after sowing.

***Table 2:*** *Overview about applied management measures including dates of sowing, inoculation and harvest at the eight sites investigated*

| Site | Sowing date | Inoculation date | Harvest date | Preceding crop | Maize variety | Type of fertilization | total fertilizer N kg/ha | fertilizer P kg/ha | N/P ratio fertilization | Weed-control |
|---|---|---|---|---|---|---|---|---|---|---|

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cazis (C)** | 27.5.2015 | 1.6.2015 | 30.9.2015 | Grass-clover mixture | Fabregas | Digested animal manure | 115.6 | 44.4 | 2.6 | chemical |
| **Ecublens (V)** | 14.5.2015 | 21.5.2015 | 21.9.2015 | Grass-clover mixture | Gottardo | Manure and urea | 211.2 | 55.0 | 3.8 | chemical |
| **Schafisheim (SH)** | 18.5.2015 | 22.5.2015 | 7.9.2015 | Grass-clover mixture | Geoxx | Slurry and di-ammonium-phosphate | 154.0 | 108.2 | 1.4 | chemical |
| **Sins (S)** | 18.5.2015 | 19.5.2015 | 8.10.2015 | Grass-clover mixture | Stabil | Manure, Ammonium-nitrate, and urea | 194.0 | 51.0 | 3.8 | chemical |
| **Unterendingen (U)** | 20.5.2015 | 27.5.2015 | 25.9.2015 | Grass-clover mixture | LG 30.222 | Manure and Ammonium-nitrate | 138.3 | 39.4 | 3.5 | chemical |
| **Wängi (W)** | 18.5.2015 | 19.5.2015 | 11.9.2015 | Grass-clover mixture | Fabregas | Slurry | 221.2 | 44.5 | 5.0 | mechanical |
| **Vordemwald (V)** | 13.5.2015 | 18.5.2015 | 8.10.2015 | Grass-clover mixture | Amadeo | Ammonium-nitrate and urea | 114.8 | 0 | NA | chemical |
| **Zofingen (Z)** | 25.5.2015 | 28.5.2015 | 9.9.2015 | Winter wheat | Fabregas | Compost and azoplum | 174.0 | 28.0 | 6.2 | mechanical |

*2.4 Sampling*

In July, approximately at the time of maize flowering, we assessed AM fungal abundance. Four soil samples were taken per plot in proximity to maize plants and pooled to form a composite sample. In the laboratory, roots were collected from the soil samples to assess AM fungal root colonization. Additionally, 6-8 leaves per plot were randomly chosen and cut from the plants for analysis of nutrient concentrations.

*2.5 Harvest*

Farmers informed about the planned harvest date. Shortly before the farmer's harvest, after approximately four to five months of plant growth, four plants from the center of each plot were cut 10 cm above soil surface, dried at 60°C and weighed. The total number of plants established per plot was noted. Subsequently, plants where threshed and ground in a cutting mill for measurement of nutrient concentrations. The root systems of the four collected plants (stem and connected roots) were removed from soil and collected. Roots were thoroughly washed with

water, cut from the stem, cut into pieces of approximately 1 cm and mixed. One subsample was stored in ethanol for later assessment of AM fungal root colonization and one subsample was frozen for DNA extraction. Four soil samples were taken from each plot in proximity of the sampled plants and pooled. Unfortunately, one farmer (site Z) had harvested the entire field and another farmer (site W) had harvested half of the experimental plots without prior information. For these plots, no aboveground plant data is available. Roots and soil samples could, however, still be sampled and analyzed from all plots. Total biomass per plot was calculated by multiplying the average dry weight of the 4 harvested maize plants with the total number of maize plants established on the respective plot.

*2.6 Plant nutrient analyses*

N concentrations of plant shoots and soils were determined with a CHNSO analyzer (Euro EA, HEKAtech, Wegberg, Germany). Plant P concentrations were determined photometrically using the molybdenum blue ascorbic acid method (Watanabe and Olsen, 1965) after dry ashing.

*2.7 AM fungal root colonization*

Root colonization by AM fungi was assessed after clearing roots with KOH and staining with an ink-vinegar mixture (Vierheilig *et al.*, 1998). A modified line-intersection method for 100 intersections was used to quantify AM fungal root colonization (McGonigle *et al.*, 1990).

*2.8 Determination of AM fungal community composition*

To assess initial AM fungal communities, we extracted genomic DNA from a 300 mg sub-sample of the composite soil samples collected before inoculation with the NucleoSpin® Soil kit (REF740780) from Macherey-Nagel (www.mn-net.com, Düren, Germany) according to the manufacturer's instructions. We quantified the AM fungal communities by single-molecule real-time (SMRT) DNA sequencing and followed the molecular and bioinformatic protocol as described in Schlaeppi *et al.* (2016) with the exception that we used 1300 bp sequence information to infer OTUs. We sequenced 3 SMRT®Cells at the Functional Genomic Centre Zurich (Zurich, Switzerland; http:// www.fgcz.ch). OTUs were clustered at a level of 97% sequence similarity.

AM fungal taxonomic identities were assigned to the sequences representing OTUs using the UNITE database (Koeljalg et al., 2013) with BLAST in the QIIME environment (Caporaso et al., 2010). OTUs assigned to the phylum 'Glomeromycotina' were subsequently queried against the AM fungal reference data set (Krueger et al., 2012) to obtain high-resolution and AM fungi-specific taxonomy. The sequence numbers of the AM fungal taxa were normalized with the total number of AM fungal reads in each sample. Reads assigned to a taxon with a relative abundance < 0.5% were removed from the dataset.

*2.9 Quantification of Rhizoglomus irregulare by qPCR*

We quantified the initial abundance of *R. irregulare* in the field soils at the start of the experiment (same DNA samples as used for AM fungal community sequencing) and in the maize roots of 3 replicate plots per treatment at harvest. Maize roots were lyophilized and ground to fine powder using a Retsch (www.retsch.com, Germany) ball mill (model MM301; settings 30 s at 30 Hz using one 1-cm steel ball). DNA was extracted from a 200 mg subsample using the NucleoSpin® Plant II kit from Machery-Nagel (www.mn-net.com, Germany) following manufacturer's instructions. DNA concentrations were determined on a Varian Eclipse Fluorescence plate reader using Quant-iTTM PicoGreen® dsDNA Assay Kit (Invitrogen) and Herring Sperm DNA (Invitrogen) as standard solution. We quantified the presence of *R. irregulare* based on quantitative real-time PCR primers developed by Alkan *et al.* (2006).

Considerable intraspecific genetic variation can occur within AM fungal species (Thiéry *et al.*, 2016). Therefore, we aimed at specifically quantifying the abundance of the *R. irregulare* strain SAF22 we used for inoculation. We validated the target sites of these primers on the ribosomal operon sequence of the *R. irregulare* strain SAF22 (**Figure S1A**) and therefore, we modified the 'VC-F' primer to match the target sequence of the strain SAF22. Inoculated roots showed an augmentation of the PCR signal compared to non-inoculated roots and the amplicon, which is produced in the inoculated root samples, ressembles the one from the pure strain DNA (**Figure S1B**). Together, this reveales that the inoculation with the *R. irregulare* strain SAF22 can be

quantified with the modified primer combination. The background signal from non-inoculated roots can be interpreted that the *R. irregulare* strain SAF22 was present at low abundance or that the primers also capture *R. irregulare* strains that are closely related to strain SAF22.

Amplification was performed in 20 µl reactions using the HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) from Solis Biodyne (www.sbd.ee, Estonia) on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler (www.bio-rad.com, USA). Reactions were performed in duplicates and each contained 4 µL qPCR mix (5X), 1 µL of each primer (10µM), 9 µL distilled sterile water and 5 µL template (5 ng DNA). The cycling program consisted of a 15 minutes' initial denaturation step at 95°C followed by 40 cycles (95°C for 15 seconds, 63°C for 40 seconds, 72°C for 20 seconds) and a 10 minutes' final extension step at 72°C. Melting curve analysis consisted of a gradient from 65°C to 95°C increasing by half degree per 10 seconds to determine the uniformity of the amplicons. Standard calibration curves were prepared from dilution series with a plasmid that carries the target sequence of the *R. irregulare* strain SAF22. For this construct, we amplified the ribosomal operon sequence (Krüger et al., 2009) from single spores of the *R. irregulare* strain SAF22 and cloned it using the 'Zero Blunt® TOPO® PCR Cloning Kit' (Invitrogen) following the manufacturer's instructions.

Raw data were exported from the qPCR cycler and imported into the LinRegPCR program to determine the $C_T$ and E values using a common fluorescence threshold for all samples (Ruijter *et al.*, 2009). Template amounts were calculated for each reaction using the individual E, averaged among the duplicates of each sample and expressed in copy numbers of the target sequence of the *R. irregulare* strain SAF22 (standard curve method, see Brankatschk *et al.* (2012)).

*2.10 Mycorrhizal growth response*

Mycorrhizal growth response (MGR) was calculated as described in Köhl *et al.* (2016) as a measure for the percentage change in maize biomass in plots inoculated with the AM fungus relative to the average biomass of control plots. Similarly, mycorrhizal N and P uptake responses

were calculated to assess the percentage change in maize N and P uptake in the inoculated plots relative to the non-mycorrhizal controls.

*2.11 Statistical analyses*

Data were analyzed at two different levels. To assess effects of the different sites and their characteristics (soil parameters, amounts of fertilizers) on native AM fungal abundance, plant biomass and nutrient concentrations, only data of the control plots were considered.

To assess effects of AM fungal inoculation and how they are affected by site, the complete dataset including control and inoculated plots was used. All statistical calculations were done in R statistical software, version 3.3.1 (R Core Team, 2016).

Differences in AM fungal root colonization and plant growth and nutrition due to site were assessed by one-way ANOVA with site as factor and using data of the control plots only.

Non-parametric Spearman's rank correlations were used to assess relationships of soil parameters and fertilizer amounts at different sites to mean percentage hyphal root colonization and mean plant biomass and nutrient parameters in control plots. Linear mixed effects models including site as random effect were used to assess relationships between percentage hyphal root colonization and plant biomass and nutrient contents in control plots.

Effects of inoculation treatments at the different sites on AM fungal and plant parameters were assessed by two-way ANOVA with site, inoculation treatment and their interaction as factors and using the complete data set. In case of significant effects, Tukey's HSD test was used for post-hoc comparisons. When ANOVA assumption could not be met, non-parametric Kruskal-Wallis tests were performed separately for each factor and interactions. Similarly, in case of significant effects, post-hoc comparisons were done by performing separate Kruskal-Wallis tests for each site.

Non-parametric Spearman's rank correlations were used to assess relationships of soil parameters and fertilizer amounts at the different sites to mean root colonization in control plots and to mean MGR per site.

Linear mixed effects models including site as random effect were used to assess relationships between the relative change in plant growth and nutrition and the relative change in root colonization and in copy numbers of *R. irregulare* strain 22 in maize roots due to inoculation. Linear mixed effects modeling was done using the R package nlme (Pinheiro *et al.*, 2016). Model fits were calculated using R package piecewise.SEM (Lefcheck, 2015). Conditional $R^2$ is reported to assess model fit.

## 3. Results

### *3.1 Native AM fungal communities*

A total of 13 different AM fungal taxa were identified at the species level, while five taxa where identified at the genus level in the soils of the different sites (Table 3). Numbers of species per site ranged from 6 (site S) to 12 species (sites U and Z). *Rhizoglomus irregulare* sequences were detected at all sites. We detected *Rhizoglomus irregulare,* strain SAF22 (or strongly related sequences) through qPCR in the soil of six sites, while in two sites (sites 'SH' and 'W') the strain was not detected (Table 3).

We correlated soil characteristics with AM fungal sequence richness to obtain insights in how soil parameters affected AM fungal diversity in the investigated soils. The number of different AM fungal sequences detected in soil at the beginning of the experiment was positively correlated to soil silt ($\rho$= 0.74, p= 0.036, Fig. S2A), and negatively correlated to sand contents ($\rho$ = - 0.76, p= 0.027, Fig. S2B). Moreover, AM fungal sequence richness was negatively related to the amount of P fertilization applied by the farmers ($\rho$=: - 0.81, p= 0.014, Fig. S2C).

AM fungal root colonization of the un-inoculated maize plants at harvest, as an indicator for abundance of the native AM fungal community, was significantly negatively correlated to total soil C ($\rho$= - 0.86, p= 0.011, Fig. 2A) and N contents ($\rho$= - 0.74, p= 0.046, Fig. 2B) and there was a marginally significant negative correlation to total soil P contents ($\rho$= - 0.69, p= 0.069. Fig. 2C).

Soil pH was positively related to AM fungal root-colonization in control plots (ρ= 0.76, p= 0.037) (Fig.2D).

***Table 3:*** *AM fungal community composition and abundance of* R. irregulare *strain SAF22 in soil at the different sites before start of the experiment.*

| | Site | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **AM fungal taxa [% relative abundance]** | **Cazis (C)** | **Ecublens (E)** | **Schafisheim (SH)** | **Sins (S)** | **Unterendingen (U)** | **Vordemwald (V)** | **Wängi (W)** | **Zofingen (Z)** |
| *Rhizoglomus irregulare* | 1.0 | 7.9 | 8.2 | 6.2 | 11.2 | 13.4 | 3.8 | 7.5 |
| *Glomus macrocarpum* | 4.6 | | | | 1.6 | 7.7 | | 27.6 |
| *Claroideoglomus claroideum* | | 11.8 | 19.0 | | 4.4 | 11.6 | 3.1 | |
| *Paraglomus brasilianum* | | | 42.5 | 36.4 | 17.6 | 3.5 | 19.1 | 3.9 |
| *Diversispora epigaea* | | 7.9 | 26.5 | 1.3 | 28.6 | | 62.0 | 24.0 |
| *Funneliformis caledonius* | | 56.2 | 1.9 | 29.3 | 5.6 | | 1.3 | 5.5 |
| *Rhizoglomus sp.* | 6.6 | | | 0.9 | | 9.6 | | 2.9 |
| *Acaulospora cavernata* | 21.0 | | | 8.0 | | | | 1.9 |
| *Ambispora fennica* | 1.0 | 9.6 | | | 3.8 | 4.9 | | 3.8 |
| *Diversispora eburnea* | | 1.7 | 1.1 | | 2.3 | 10.2 | | 2.6 |
| *Rhizoglomus intraradices* | 28.5 | | | | | 2.5 | 0.7 | |
| *Scutellospora sp.* | | | | | | | | 2.5 |
| *Claroideoglomus sp.* | | | | | 2.3 | | | |
| *Diversispora sp.* | 4.0 | | | | 3.7 | | | |
| *Funneliformis coronatus* | 32.9 | | | | | 34.9 | | |
| *Funneliformis mosseae* | | 4.5 | | 17.8 | 12.6 | | | 1.6 |
| *Glomus sp.* | | 0.6 | | | 6.3 | 1.5 | | |
| *Scutellospora gilmorei* | | | | | | | 9.9 | 15.6 |
| **No. of taxa** | 8 | 8 | 6 | 7 | 12 | 10 | 7 | 12 |
| **qPCR** | | | | | | | | |
| *Abundance of R. irregulare* strain SAF22 in soil [sequence copy numbers x$10^{-5}$] | 12.48 | 3.07 | ND* | 21.96 | 1.29 | 1.84 | ND* | 2.55 |

* not detected

*3.3 Establishment of the inoculated fungus*

In root samples collected in July during maize flowering, we detected an overall significant increase in hyphal (F= 6.09, p= 0.016, Fig. S3A) and vesicular root colonization (Kruskal-Wallis: $chi^2$= 36.2, p< 0.001, Fig.S3B) in plots inoculated with *R. irregulare* strain SAF22.

At maize harvest, however, inoculation with *R. irregulare* only enhanced the average AM fungal root colonization compared to the control plots in one out of the 8 sites investigated (Site:Inoculation interaction, F= 3.9, p= 0.001, Fig.3A). Conducting pairwise comparisons, the difference was only found to be significant at site 'U'. Here, average root length colonized by AM fungi was 93.5% in the inoculated maize plants, while it was 56% in the control plants. This corresponds to a 66% increase in total root colonization. In 4 out of 8 sites, there was an average increase in vesicular root colonization in the inoculated plots (Site:Inoculation interaction, F = 77.3, p< 0.001, Fig.3B). The differences between treatments were, however, only significant at sites 'U' and 'W'. Similarly, arbuscular root colonization was significantly increased at sites 'U' and 'W' (Fig. 3C).

Inoculation enhanced the average abundance of *R. irregulare* strain SAF22 in maize roots at harvest at all sites as determined by qPCR (Fig. 3D). In five of eight sites investigated, this increase was significant. In seven of eight sites, we detected *R. irregulare* strain SAF22 in the roots of the control plots, indicating that this strain, or closely related strains had already been present in the field before inoculation.

We defined the establishment success of the inoculated fungus as the percentage increase of *R. irregulare* strain SAF22 sequence copy numbers in maize roots of the inoculated plots compared to the control plots (Fig. 3D). We found a strong positive relationship between total soil P contents and establishment success of the fungus ($\rho$ = 0.81, p=0.022, Fig. 4A). Positive relationships between establishment success and organic C and available P were also found, although less strong (organic C: $\rho$ = 0.460, p=0.021; available P: $\rho$ = 0.465, p=0.022). Initial abundance of *R. irregulare* strain SAF22 expressed as the number of AM fungal sequences of this strain detected in soil before inoculation had no influence on the establishment success of the inoculated fungus. However, there was a negative relationship between the site-specific root colonization, defined as the root colonization of maize plants in the control plots at harvest and establishment success

of the inoculated fungus (Fig. 4B). This indicates that the inoculated fungus established better at plots where the native level of AM fungal abundance was lower.

*3.4.2 Effects of inoculation on maize performance*

No overall effect of AM fungal inoculation on total maize dry weight per plot was observed across sites (Fig. 5A). We calculated the mycorrhizal growth response (MGR) as a measure for the effect of AM fungal inoculation on maize biomass relative to the control treatments. MGR varied from slightly negative (-4.9 to -2.5% at side E, S and SH) to positive (+14% at site U) (Fig. 5B). For site 'U', the MGR was significantly greater than 0 as assessed by a one-sample t-test ($t= 3.24$, $p= 0.023$). Analogously, for mycorrhizal P response (MPR), the relative change in plant P uptake compared to the control treatments, tended to be positive at 5 and negative at 2 sites and was also significantly enhanced at site U ($t= 4.95$, $p= 0.004$, data not shown). Both sites showing the highest average MGR and MPR (sites 'U' and 'W') were the same sites showing a significant increase in vesicular and arbuscular root colonization upon inoculation (see Fig. 3A-C). Only minor effects of inoculation on plant nutrient concentrations were observed (Tables S2 and S3). There was a significantly positive relationship between the establishment success of *R. irregulare* strain SAF22 and relative increase in plant P uptake (MPR) in inoculated plots compared to control plots ($R^2= 0.29$, $p= 0.032$, data not shown). We tested, whether the direction and extent of MGR depended on specific site characteristics (i.e. soil physical and chemical properties and management). We found that the amount of P fertilization applied by the farmers was significantly negatively correlated to mean MGR per site (Spearman`s rho= 0.36, $p= 0.029$, Fig. 6). No further variables assessed showed significant effects on MGR.

## 4. Discussion

*4.1 Summary*

This study investigated community abundance and composition of plant symbiotic arbuscular mycorrhizal fungi in Swiss agricultural fields. We assessed whether a well-characterized AM

fungal strain inoculated into these fields can successfully establish in plant roots and can generate agronomic benefits for maize production. Furthermore, we identified environmental, management and biological factors affecting establishment success of the introduced fungus.

Abundance and sequence richness of native AM fungal communities varied with site conditions, such as soil texture, pH, and nutrient status. Successful establishment of the inoculated AM fungal strain varied with native AM fungal abundance and soil P contents. While there was a negative trend of native AM fungal abundance with increasing soil P contents, we found significantly positive relationships between establishment success of the inoculated fungus and soil P contents, as well as significantly negative relationships between AM fungal establishment success and abundance of native AM fungal communities. Taken together, these results imply that i) abundance and sequence richness of native AM fungal communities is negatively affected by soil N, C and P contents and ii) low abundance of the native AM fungal communities increases chances of successful AM fungal inoculation.

*4.1 Abundance of native AM fungal communities*

Native AM fungal abundance was higher in finer textured soils, in soils with lower total nutrient concentrations and with higher pH values. Similarly, AM fungal sequence richness was higher in finer textured soils and in soils with lower total P concentrations. These findings agree with earlier observations that mycorrhizal abundance is reduced in acidic environments (van Aarle *et al.*, 2002; Goransson *et al.*, 2008). It is well known that high soil nutrient levels have adverse effects on AM fungal abundance and functioning (Treseder, 2004). Here, we confirm that AM fungi are more abundant in soils of lower fertility and higher pH across farmers' fields distributed across Switzerland, mostly on soils possessing high nutrient levels.

Soil Phosphorous concentration is known to affect AM fungal abundance. While under low soil P conditions an increase in P concentration can enhance AM fungal abundance, under higher soil P conditions an increase in P concentration negatively affects AM fungal abundance (Abbott L *et al.*, 1984; Treseder and Allen, 2002). Seven of the 8 soils investigated were classified as

sufficiently or strongly supplied with P, while one soil (site ‘C’) was rather poor in P, according the Swiss Principles of Fertilizer Application in Arable and Forage Cultivation (Flisch *et al.*, 2009). Therefore, a negative relationship between soil Phosphorous and AM fungal abundance can be expected under the conditions investigated.

*4.2 Factors determining establishment success of the inoculant*

With the abundance of native AM fungi diminishing due to increasing soil P levels, establishment success of the inoculated AM fungal strain increased. The negative relationship between native AM fungal abundance and establishment success could be explained by reduced competition effects with a less abundant native community (Niwa *et al.*, 2018). Reduced abundance of native AM fungal communities should increase niche space available for newly introduced AM fungi. In a study investigating effects of AM fungal inoculations on leek plants, Hamel *et al.* (1997) found that leek growth responded positively to inoculations only in soil with high P contents, while the response was absent in soils with lower P contents. They concluded that high soil P levels may have led to reduced or inefficient native AM fungal communities, therefore increasing inoculation benefits. Although our study did not find consistent plant growth responses, our findings support the claim, that adverse effects of soil P contents on native AM fungal communities may increase positive effects of AM fungal inoculations.

*4.3 Competition between inoculant and native communities*

Inoculation enhanced total root colonization at only one site, while colonization with vesicles and arbuscles was significantly enhanced at two of the eight sites. In contrast, the qPCR analysis using primers specifically amplifying the applied *R. irregulare* strain SAF22 indicated that the inoculation led to a significant increase in abundance of the inoculated strain in five of eight soils. The fact that this increase was not reflected in root colonization indicates on one hand that the overall colonization potential of the plant roots was apparently already reached at most sites (Hamel *et al.*, 1997); on the other hand, it indicates that the inoculated strain might have competed efficiently with native AM fungal communities, replaced some native fungal community members

and became integrated in the local community. In roots where DNA copy numbers did, but colonization did not increase, the inoculated fungus must have occupied root niche space by replacing parts of the native AM fungal community. The work by Schlaeppi *et al.* (2016) also showed that *R. irregulare* strain SAF22 can largely replace native AM fungal strains within plant roots. These results imply that molecular tools become necessary to reliably assess the establishment of inoculated AM fungi into systems with an already established AM fungal community, as establishment success may not simply be reflected in higher colonization levels. Moreover, these findings raise concerns about the biosafety of AM fungal inoculations and potential negative effects on native communities (Machado *et al.*, 2016; Bender *et al.*, 2017).

In the present study, although root colonization seemed not to be a reliable indicator for successful establishment of the inoculant, positive MGR was only observed in fields where root colonization also increased.

These findings imply that our inoculated strain might not be superior in its plant-growth-promoting capabilities relative to already existing communities, but that under conditions with potential for increases in root colonization, inoculation can provide additional benefits by enhancing overall AM fungal abundance in plant roots.

Recent work by Niwa *et al.* (2018) concluded that the dominance of an inoculated fungus over native AM fungal communities is a prerequisite for plant growth promoting effects in soy bean fields. However, their results also showed that not in all systems dominance of their inoculant over native AM fungal communities led to plant growth benefits. However, in their study, AM fungal root colonization was not assessed, and their conclusions are solely based on molecular analyses of AM fungal dynamics in roots. It remains, therefore, unclear whether the observed dominance of the inoculated strain in this study led to increases in root colonization or not.

Depending on local conditions, plant variety, and potential other abiotic and biotic factors, there might be a maximum fraction of root length potentially available for colonization by AM fungi.

If native communities can fill available root niche space, AM fungal inoculations may not lead to increased colonization levels. However, as suggested by our data and data of Schlaeppi *et al.* (2016), inoculated AM fungi can compete with native AM fungi for available niche space and replace native communities to a certain extent.

If native communities are deprived, e.g., through over-intensive agricultural management practices, root colonization potential might not be fully exploited. Inoculated AM fungi are, hence, more easily able to fill out available space, increase root colonization levels, and, as suggested by our data, enhance plant responses to inoculations.

Overall, our results suggest that the niche space available to the inoculated fungus (i.e. field carrying capacity for root colonization) might be a stronger predictor for enhanced plant benefits than its' suitability to specific soil conditions (i.e. species compatibility, Verbruggen *et al.* (2013)).

*4.4 Effects on crop yield*

AM fungal inoculation only in parts showed agronomic benefits. The extent of MGR was significantly negatively correlated to the amount of P added with fertilizer (Fig. 6), suggesting that lower P fertilization levels might promote positive plant responses to inoculations. A meta-analysis by Lekberg and Koide (2005) reported a negative relationship between available soil P and MGR across various field and glasshouse trials. The authors noted that at low P availability, there was a high variability among the MGR data indicating that under such conditions factors other than P become dominant in affecting MGR. Consistently, MGR at site 'V' -with no P fertilizer applied- showed the highest variation in our study (Fig. 6). However, this site also comprised two replicates with the highest MGR across the whole study and further experimentation is necessary to reveal the causes for this high variation (e.g. experimental constraints or high variability (and low predictability) as suggested by Lekberg & Koide (2005)). Lekberg and Koide (2005) concluded from the high variation in MGR at low P levels that these conditions do not necessarily generate the greatest growth responses but provide conditions where the greatest growth responses could potentially occur.

We analyzed soil P contents only at the beginning of the experiment at the time of inoculation and we didn't find a direct relationship between P availability and MGR. However, all P fertilizer in this study was applied in organic form. It can be expected that organic fertilizers increase P solution levels in soil over the growing season (Singh and Jones, 1976), providing conditions of relatively higher available soil P content with higher fertilization intensity during plant development. Altogether, our data indicate that Phosphorous plays a central role in shaping the relationship between native AM fungal abundance and diversity patterns and the establishment and effectiveness of inoculated AM fungal strains. While total soil P content affects native AM fungal abundance and establishment, the growth response to inoculation depends on amounts of P fertilizer applied.

## 5. Conclusions

Taken together, our results imply that there exists a trade-off between supporting native AM fungal communities and creating conditions conducive for high establishment success of inoculated fungi. Under conditions where native AM fungal communities were less abundant, establishment success was higher. However, successful establishment only provided agronomic benefits when it also led to increases in root colonization. Further research with a wider range of field sites will be necessary to identify factors that determine the extent of root colonization possible in a certain root environment to be able to predict whether AM fungal inoculation has the potential to enhance root colonization. Moreover, the long-term persistence of fungal inoculants in field settings will require further investigation (Pellegrino *et al.*, 2012). While the form and large amount of inoculum applied in the present study serves to inform scientific investigations, it may not be economically viable in an applied context, especially for staple crops like maize. However, practically more applicable approaches to produce and apply inoculum exist (Ijdo *et al.*, 2011; Ceballos *et al.*, 2013; Hijri, 2016). Together with sound understanding about the conditions under which AM fungal inoculations provide benefits, the potential of such approaches could be fully exploited to

enhance the sustainability of arable systems. Co-inoculations of AM fungi with other groups of beneficial soil organisms could provide further benefits for agricultural production (Imperiali *et al.*, 2017).

Based on our results, we propose that to assess the potential for successful AM fungal inoculation (i.e. generating plant growth promoting effects) at a certain field site, root colonization potential should be assessed in a standardized manner (see e.g. Plenchette *et al.* (1989)). Further research is needed to assess the potential for large-scale application of such an approach in commercial agriculture.

Overall, the strategy of choice would be to promote abundant and diverse native AM fungal communities that fill available niche space and provide agronomic benefits without the costs and risks associated with inoculating non-native fungal strains into agricultural fields. However, if native AMF communities are in a suboptimal state, e.g. through over-intensive agricultural management, inoculation with efficient AM fungal strains can be a strategy to restore internal ecosystem processes to support agricultural production in an environmentally friendly way.

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717 **Figure captions**

718

719 **Figure 1:** Map of Switzerland illustrating the locations of the 8 maize fields used in this study.

720

721 **Figure 2:** Relationships of average native AM fungal abundance expressed as percentage maize
722 root length colonized at harvest in the control plots to soil chemical parameters. ((A) Soil organic
723 carbon, (B) Total soil nitrogen, (C) Total soil phosphorus, (D) soil pH) across 8 different Swiss
724 arable fields. Non-parametric Spearman's rank correlations were performed. Spearman's rho (ρ)
725 and the corresponding significance levels are shown in the respective graphs. Grey points
726 indicate average values per site, black dots show the scatter of AM fungal root colonization in the
727 different control plots per site.

728

729 **Figure 3:** Abundance of AM fungal structures in maize roots either inoculated with AM fungi or
730 control inoculum in a range of Swiss maize fields at harvest. (A) total root colonization, (B)
731 vesicular root colonization (C) arbuscular root colonization and (D) sequence copy numbers of
732 the inoculated *R. irregulare* strain SAF22. ANOVA results for effects of Inoculation and
733 Inoculation:Site interaction are given in the respective graphs. Pairwise comparisons between the
734 inoculation treatments for each Site were derived by performing Tukey's HSD test. Statistical
735 significance is indicated as ns= not significant, *= $p<0.05$, **= $p< 0.01$, ***= $p< 0.001$. nd= not
736 detected. Error bars represent means ± 1SE.

737

738 **Figure 4:** Factors affecting establishment success of *R. irregulare* strain SAF22 in a range of
739 Swiss maize fields. (A) Relationship between total soil phosphorus and establishment success
740 of the inoculated fungus (i.e. the percent change in *R. irregulare* strain 22 copy numbers due to
741 inoculation). Non-parametric Spearman's rank correlation was performed. Spearman's rho (ρ)

and the corresponding significance level are shown in the graph. Grey points indicate average values per site, black dots show the scatter of establishment success in the different plots per site. (B) Relationship between site-specific root colonization, defined as the root colonization of maize plants in control plots at harvest and the establishment success of the inoculated fungus. Relationship was tested with mixed effects model accounting for the effect of different sites as random effect. Conditional $R^2$ and the significance of the relationships are given in the plot.

**Figure 5**: Effects of inoculation with *R. irregulare* Strain SAF22 (light grey) or a non-mycorrhizal control inoculum (dark grey) at 7 different sites across Switzerland on (A) aboveground maize biomass in experimental plots (1 $m^2$) and (B) Mycorrhizal growth response, showing the percentage change in biomass of inoculated plants relative to the average biomass of un-inoculated plants. For (A), ANOVA results for the effects of Inoculation and the interaction between Inoculation treatment and site are given; for (B), significant differences from 0 were assessed by one-sided t-tests and test results are given above respective bars as ns $p > 0.1$ and * $p < 0.05$. For one of the 8 sites investigated (site 'Z'), no biomass data was available. Bars represent means of 6 replicated plots per site (site "W": 3 replicated plots per site) ± 1SE.

**Figure 6:** Relationship between the total amounts of P fertilizer applied by the farmers and the mean mycorrhizal growth response (MGR) of maize plants at each of 7 famer-managed fields across Switzerland. The relationship was tested using non-parametric Spearman's rank correlation. Test results are given in the plot. Grey points show mean MGR per site ± 1SE, black points show scatter among single replicates. Letters specify the identity of the different sites according to Table 1.

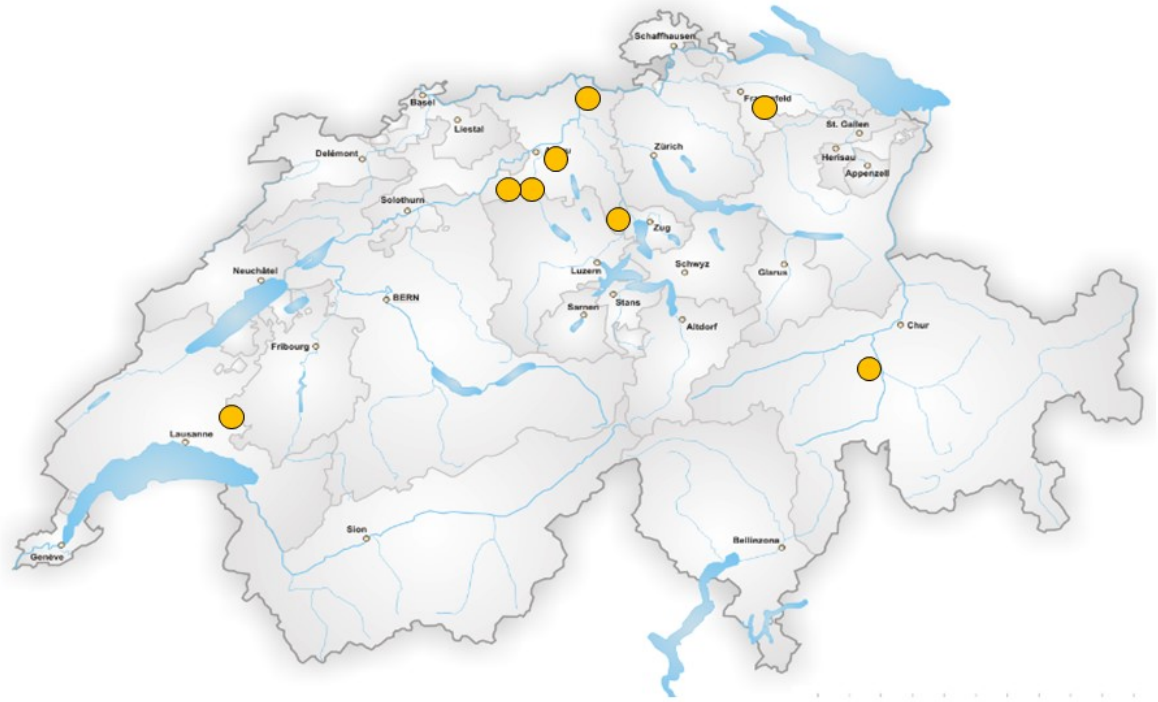

Schaffhausen
Basel
Liestal
Delémont
Zürich
St. Gallen
Herisau
Appenzell
Solothurn
Zug
Neuchâtel
Luzern
Schwyz
Glarus
BERN
Sarnen
Stans
Altdorf
Chur
Fribourg
Lausanne
Sion
Genève
Bellinzona

Figure 1

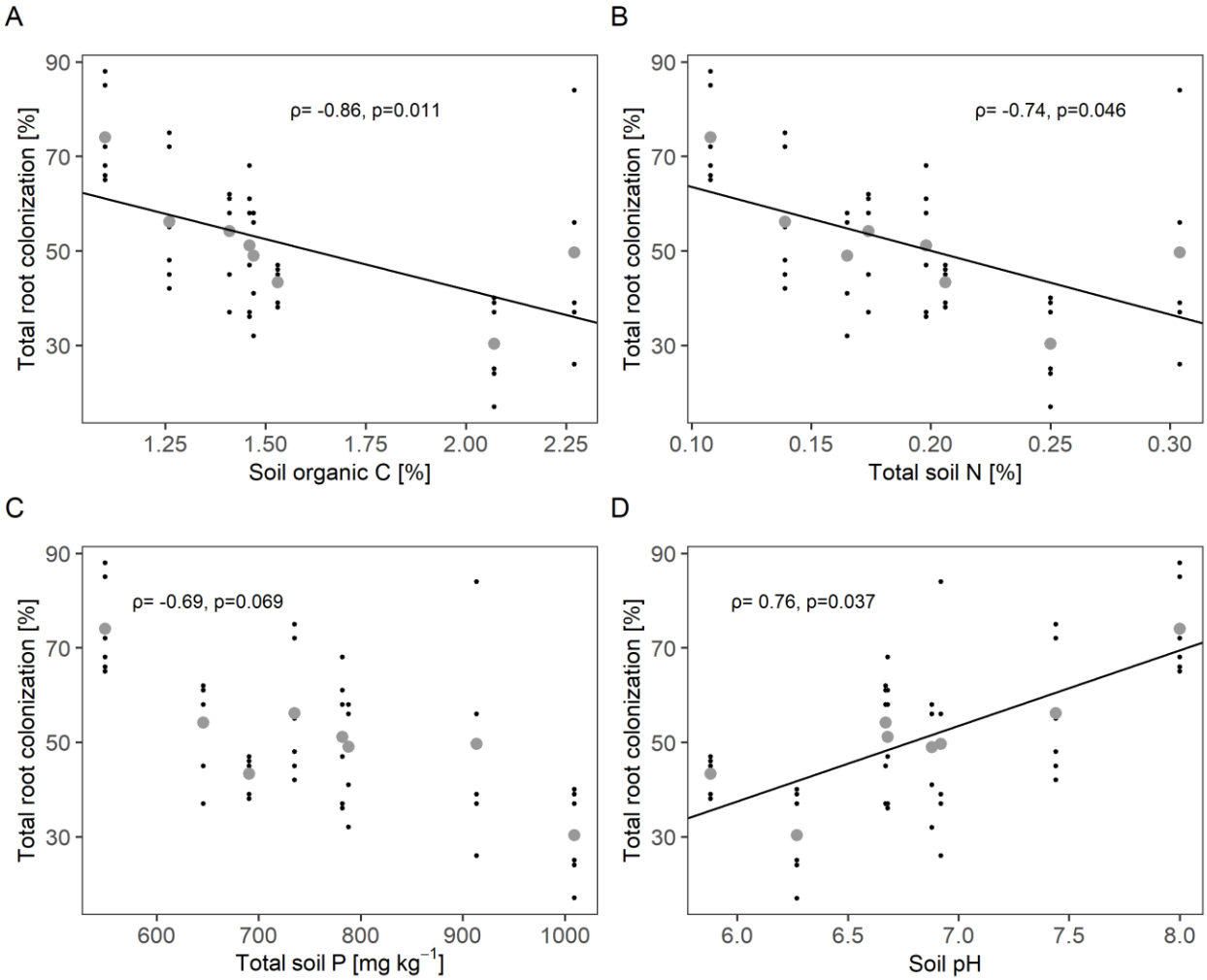

A
B
C
D
Total root colonization [%]
Soil organic C [%]
Total soil N [%]
Total soil P [mg kg$^{-1}$]
Soil pH
ρ= -0.86, p=0.011
ρ= -0.74, p=0.046
ρ= -0.69, p=0.069
ρ= 0.76, p=0.037

Figure 2

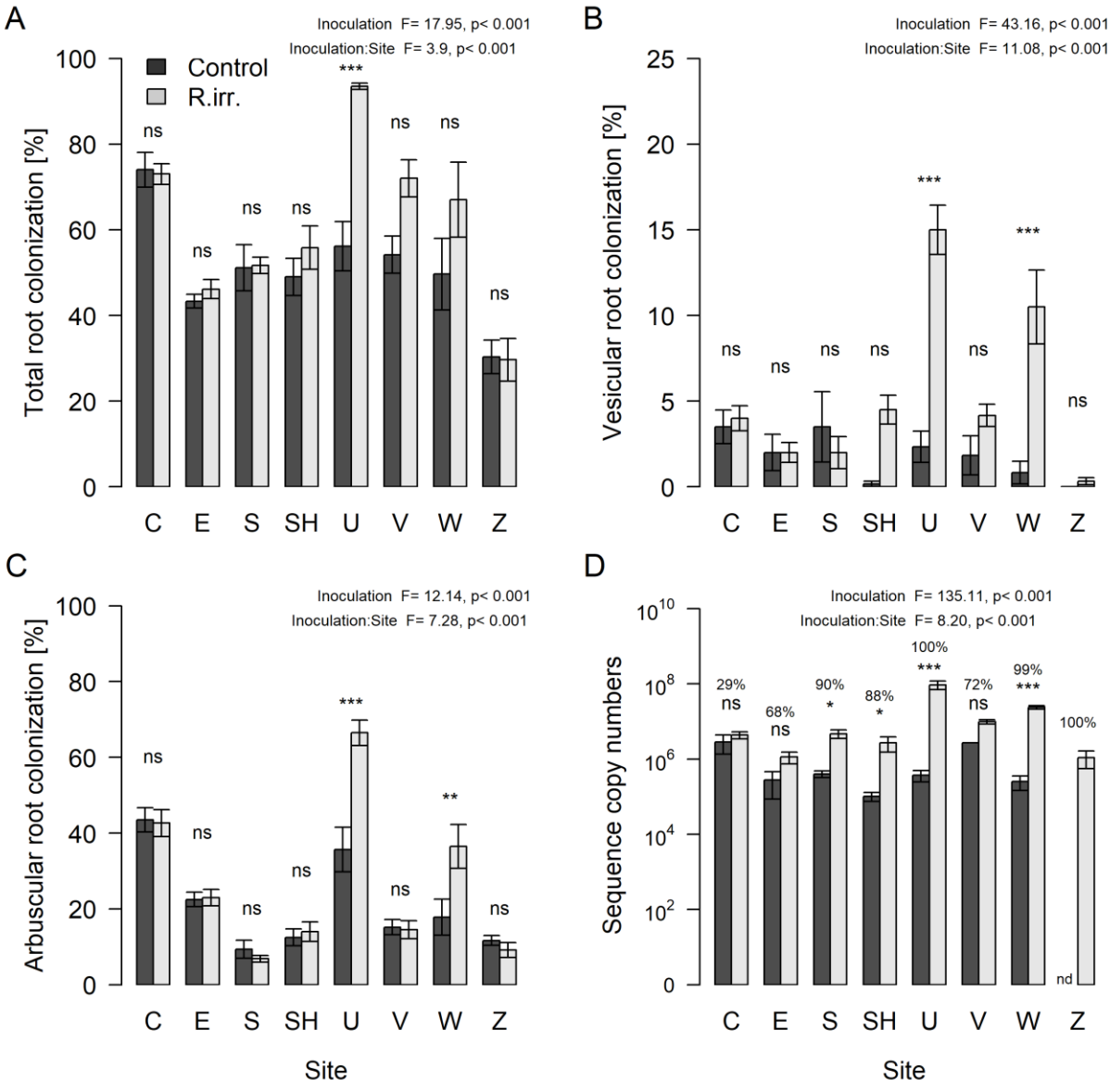

A
Inoculation F= 17.95, p< 0.001
Inoculation:Site F= 3.9, p< 0.001
Control
R.irr.
Total root colonization [%]
B
Inoculation F= 43.16, p< 0.001
Inoculation:Site F= 11.08, p< 0.001
Vesicular root colonization [%]
C
Inoculation F= 12.14, p< 0.001
Inoculation:Site F= 7.28, p< 0.001
Arbuscular root colonization [%]
D
Inoculation F= 135.11, p< 0.001
Inoculation:Site F= 8.20, p< 0.001
Sequence copy numbers
C E S SH U V W Z
Site

Figure 3

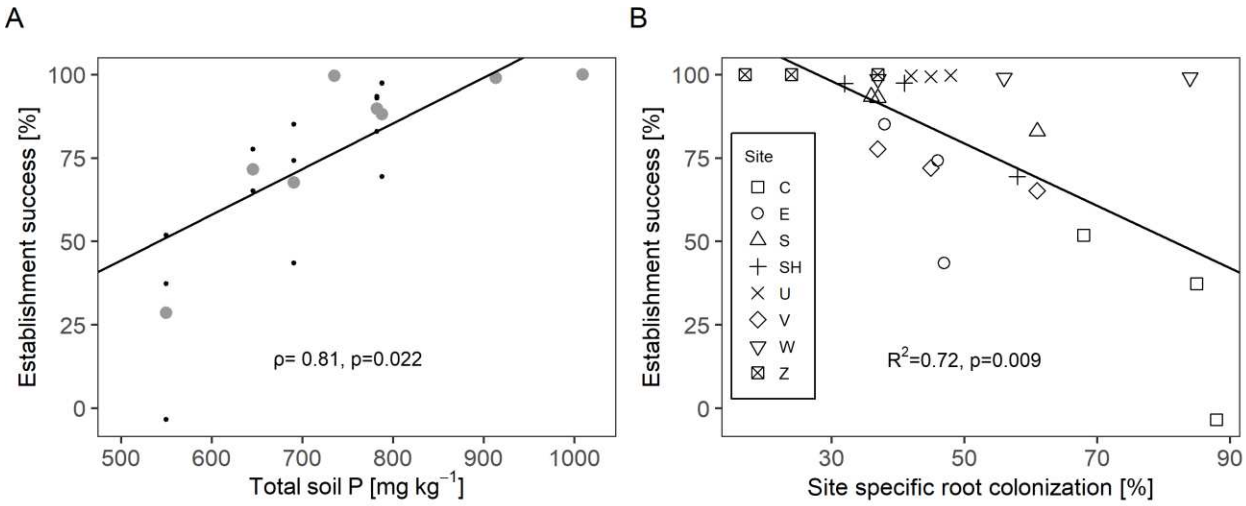

A
B
Establishment success [%]
100
75
50
25
0
500
600
700
800
900
1000
Total soil P [mg kg$^{-1}$]
ρ= 0.81, p=0.022
Establishment success [%]
30
50
70
90
Site specific root colonization [%]
$R^2$=0.72, p=0.009
Site
C
E
S
SH
U
V
W
Z

Figure 4

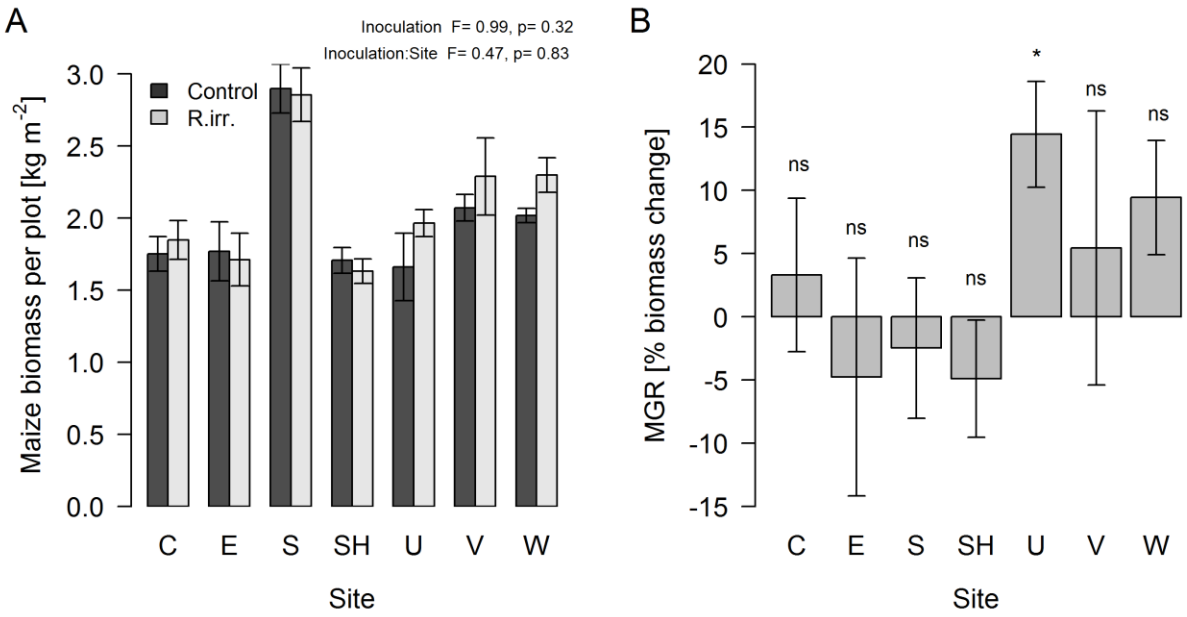
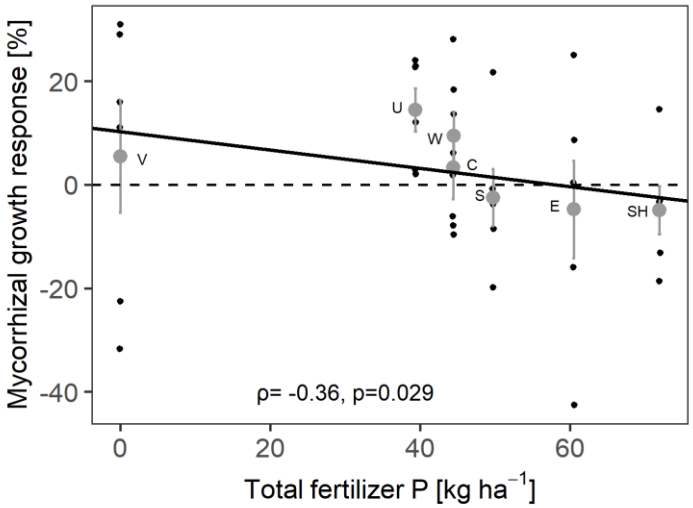

A
Inoculation F= 0.99, p= 0.32
Inoculation:Site F= 0.47, p= 0.83
Control
R.irr.
Maize biomass per plot [kg m-2]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C
E
S
SH
U
V
W
Site
B
MGR [% biomass change]
20
15
10
5
0
-5
-10
-15
ns
ns
ns
ns
*
ns
ns
C
E
S
SH
U
V
W
Site

Figure 5




861

862 Figure 6